Section IV. Remarks

Acknowledgement of Allowance of Claims 1-23 and Allowability of Claims 29-38

Applicants hereby acknowledge the allowance of claims 1-23 and the allowability of claims 29-38, as indicated in the July 1, 2004 Office Action.

Submission of Additional References In Reply to the Examiner's Requirement

Per the Examiner's request in the July 1, 2004 Office Action, a copy of the European Patent No. 0106662 as listed in the July 13, 2001 Information Disclosure Statement, a copy of the journal article by Horst Ebel as cited on page 4 of the instant specification, and a Supplemental Information Disclosure Statement citing the Ebel article are enclosed herewith in **Appendix C**.

Corrections to the Drawings

Figures 1A and 1B has been objected to by the Examiner in the July 1, 2004 Office Action under 37 C.F.R. 1.84(p)(5) for containing a reference numeral 14 that is not mentioned in the description of the present application.

In response, Applicants have hereby amended the instant specification to provide description for the reference numeral 14 in Figures 1A and 1B.

Figures 2, 9, and 10 have been amended by adding a legend "Prior Art" therein, consistent with the Examiner's requirement in the July 1, 2004 Office Action.

Figures 5-8 have been substituted with replacement drawing sheets, in compliance with the requirements of the June 10, 2004 Notice of Draftsperson's Patent Drawing Review.

Amendments to the Specification

The instant specification has been amended to indicate the updated status of U.S. Patent Application No. 09/365,063, consistent with the Examiner's requirement in the July 1, 2004 Office Action.

Amendments to the Claims

Claims 30-43, which contain two separate and distinct claims numbered 30 and two separate and distinct claims numbered 35, have been hereby renumbered as claims 30-45, consistent with the Examiner's suggestion in the July 1, 2004 Office Action.

Claims 5 and 28 have been hereby amended by replacing the limitation "in a range of from about" with a new limitation "ranging from about," thereby overcoming the Examiner's objection in the July 1, 2004 Office Action.

The originally filed claims 31 and 36-38, which were rejected by the Examiner under 35 U.S.C. §112 for being indefinite, have been hereby (1) renumbered as claims 32 and 38-40 and (2) amended to respectively depend from the renumbered claims 31 and 37.

Such claims 32 and 38-40 as renumbered and amended are definite and in compliance with the requirements of 35 U.S.C. §112, second paragraph.

Response to the Obviousness Double-Patenting Rejection of Claims 24-28, 39-40, and 41-43

In the July 1, 2004 Office Action, the Examiner rejected claims 24-28, 39-40, and 41-43 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, 2, 10, and 27 of Kurtz et al. U.S. Patent No. 6,301,330 in view of Maris U.S. Patent No. 6,038,026.

Further, the Examiner has indicated that claim 29, which depends directly from the rejected claim 24, would be allowable if rewritten in independent form.

In response, Applicants have hereby incorporated all the limitations of such allowable claim 29 into the rejected claim 24 and correspondingly cancelled claim 29.

Claim 24 so amended, together with its dependent claims 25-28, 39-40, and 41-43, therefore overcome the judicially created obviousness-type double patenting doctrine and are allowable.

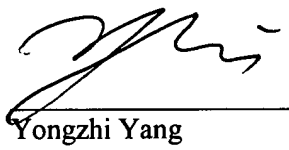
CONCLUSION

Based on the foregoing, pending claims 1-28 and 30-45 as cancelled/amended/renumbered herein are in form and condition for allowance. Issue of a Notice of Allowance for the application is therefore requested.

In relation to the renumbering of claims 30-43 to 30-45 and the cancellation of claim 29, the Office is hereby authorized to charge an additional claim fee in the amount of $18.00 to the credit card specified in the Credit Card Payment Form enclosed herewith. The Office is further authorized to charge any fees deemed necessary for entry of this Response to Deposit Account 08-3284 of Intellectual Property/Technology Law.

If any issues remain outstanding, incident to the formal allowance of the application, the Examiner is requested to contact the undersigned attorney at (919) 419-9350 to discuss same, in order that this application may be allowed and passed to issue at an early date.

Respectfully submitted,



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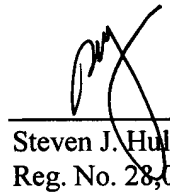

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APPENDIX A

Marked-up Copy of the Amended Figures 2, 9, and 10

with Red-Ink Sketches Showing Changes Made

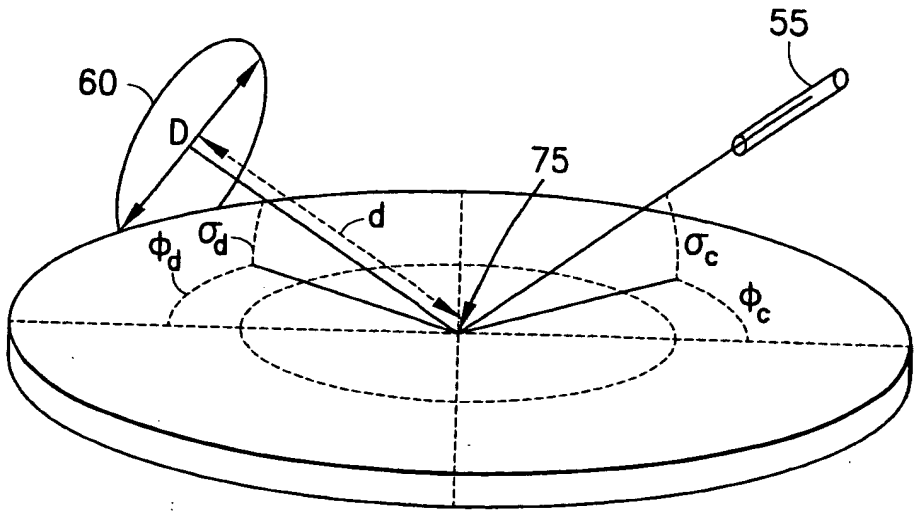

Figure 2
(PRIOR ART)

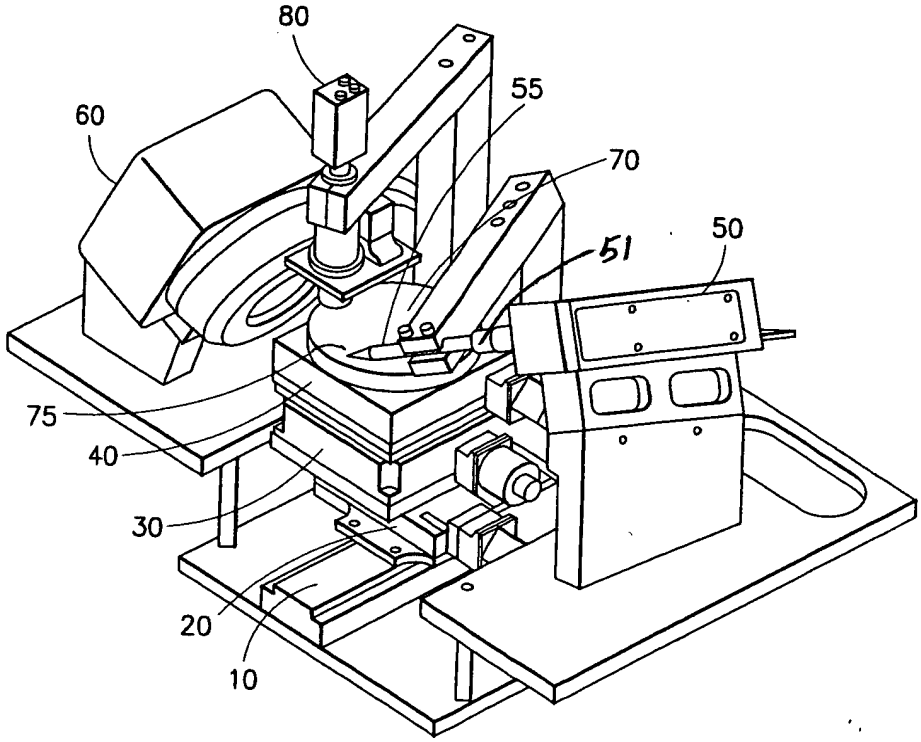

Figure 3

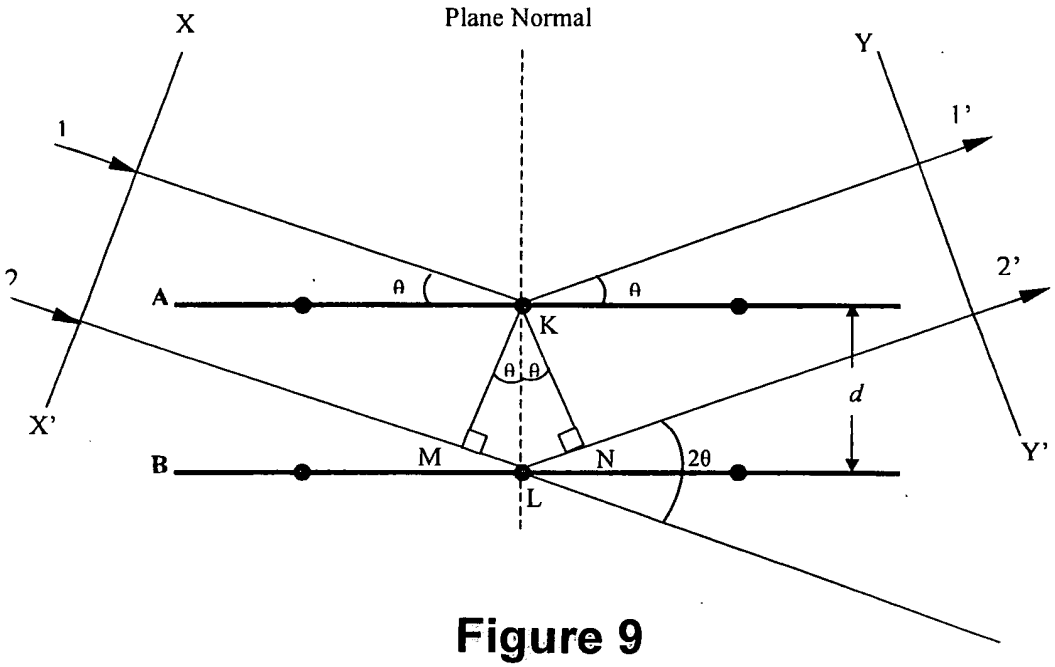

Figure 9

(PRIOR ART)

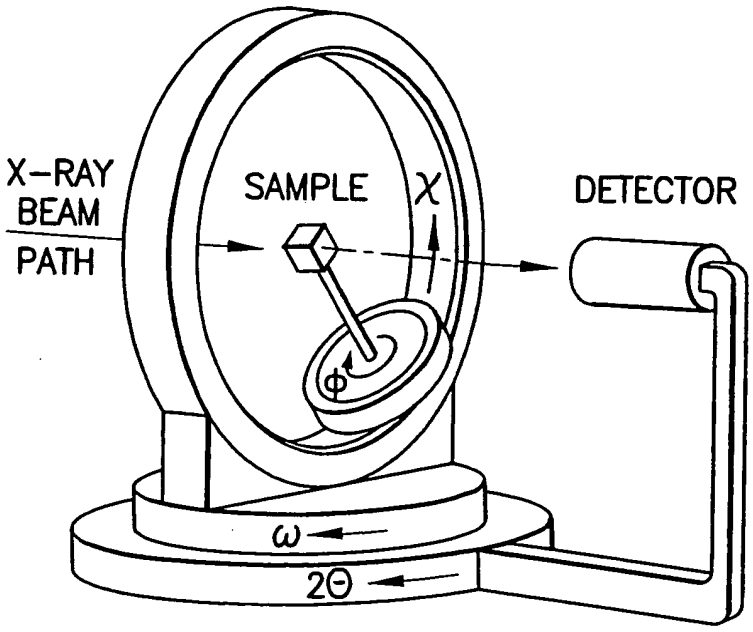

Figure 10

(PRIOR ART)

APPENDIX B

Replacement Drawing Sheets Containing Figures 2 and 5-10 as Amended

APPENDIX C

Additional References and Supplemental Information Disclosure Statement Form

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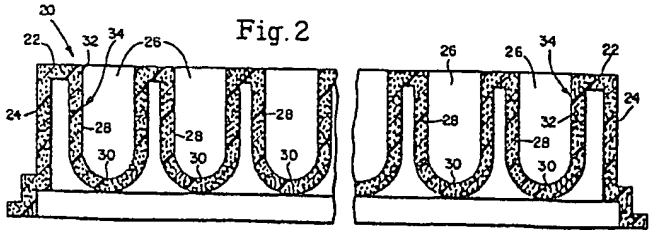
(54) **Non-fluorescent vessels for holding test samples in fluorescent assays.**

(57) A vessel (20) for holding test samples to be measured for fluorescence is formed from a material having a native fluorescence. It is provided with a barrier (34) which blocks penetration or at least reduces the extent of penetration of an exciting light into the vessel's fluorescable material to prevent fluorescent excitation of the vessel or at least reduces the extent to which the vessel is fluorescently excited when exposed to an exciting light during the fluorometric measurement.

Fig.2



EP 0 106 662 A2

0106662

NON-FLUORESCENT VESSELS FOR HOLDING TEST SAMPLES IN FLUORESCENT ASSAYS

This invention generally relates to laboratory equipment for use in conducting fluorescent assays and is particularly concerned with vessels for holding test samples to be measured for their fluorescence.

Fluorescent assays are now commonly used for diagnostic and research purposes to detect and measure the quantity of a wide variety of immunological and non-immunological substances. In fluorescent immunoassays, the test sample may be prepared for fluorometric measurement in a variety of different ways.

For example, a fluorescently labeled reactant may be immunologically reacted with the immunological substance of interest which is usually directly or indirectly immobilized on a solid phase. Following separation of the nonspecific substances which are not attached to the substance of interest, the fluorescence of the reaction product is measured to determine the amount of the substance of interest. In another type of fluorescent immunoassay, an enzyme labeled reactant is attached to the immunological substance of interest, and a fluorogenic substrate is catalyzed by the enzyme label to yield a fluorescent product which can be fluorescently measured to determine the quantity of the substance of interest.

In carrying out fluorescent assays of the foregoing type and also other types, microtest plates (or microtitration plates, as they are also called) and strips of microtest wells

are often used. Microtest plates are formed with a multiplicity of wells which are joined together in a molded one-piece structure for containing microliter quantities of fluid samples. Examples of a microtest plate and microtest wells in strip form are described in U.S. Patent No. 4,154,795 which issued to A.C. Thorne on May 15, 1979.

The use of microtest plates and microtest strips of wells in fluorescent and other types of assays offers several important advantages. First, they permit the mass preparation of a large number of test sample solutions at the same time. Second, they are more convenient to handle as compared with individual test tubes. Third, they can easily and inexpensively be washed. Fourth, they are inexpensive and disposable. Fifth, they are customarily formed from plastic materials which are not fragile like glass. Sixth, they can be made from a material having an ability to attract certain molecules such as protein molecules so that they can serve as a solid phase in an immunoassay. Polystyrene and polyvinyl chloride are commonly used for this purpose and exhibit acceptable protein binding properties for attracting protein molecules.

The use of plastic materials permits the microtest plates and strips to be manufactured by low cost, mass production molding techniques.

For fluorescent assays, however, molded microtest plates and strips of the type described above have a serious drawback in that the moldable materials customarily used for low cost manufacture exhibit a substantial level of native fluorescence, particularly at the exciting light wave lengths

commonly employed in fluorometers. When used to hold a test sample in a fluorometer for making a fluorometric measurement they therefore will unavoidably be excited along with the test sample by the fluorometer's exciting light. As a result, the microtest plates, strips or their individual wells will fluoresce to produce spurious light emissions which interfere with and impair an accurate measurement of the intensity of the light emitted from the excited test sample itself. These spurious light emissions have the objectionable effect of creating a noise signal in the fluorometer's detector to significantly reduce the signal-to-noise ratio.

One solution to the foregoing problem is to equip the fluorometers with special, sample-holding vessels made of non-fluorescent or low-fluorescing materials such as quartz or certain kinds of glass. In addition, optical grade Teflon has been suggested as a non-fluorescing material for making fluorometer flow cells in U.S. Patent No. 4,008,397 which issued to J.J. Zdrodowski on February 15, 1977. Also, an Italian company called Kartell is marketing a molded cuvette of undisclosed plastic material which is claimed to have a sufficiently low level of fluorescence to make it suitable for holding samples in a fluorometer. Although the level of fluorescence of the Kartell cuvette is lower than that of clear polystyrene, it nevertheless is significant.

The foregoing cuvettes and test tubes share a common drawback in that they are each capable of holding only a single test sample. Vessels of this type are therefore less convenient to work with as compared with microtest plates or strips. More

particularly, they are not suitable for the mass preparation of test samples as with microtest plates and strips. In addition, they are more difficult and more expensive to wash in the course of performing the various steps in an immunoassay.

Furthermore, quartz and glass are not suitable for making the intricately shaped microtest plates and strips because the manufacturing costs would be prohibitively high. In addition, the protein binding properties of quartz and glass are inferior to the protein binding properties of polystyrene and other plastics which are customarily used for making microtest plates and strips. They therefore are not as suitable as polystyrene and other plastics for defining a solid phase in an immunoassay. Finally, they are fragile and are not intended to be disposable.

Because of the disadvantages associated with cuvettes or tubes, it is often desirable and sometimes necessary to prepare the test samples in a standard microtest plate or strip and then to transfer the samples to the cuvettes or tubes for measurement. Transferring the test samples is time consuming, inconvenient and increases the cost of the fluorescent assay.

From the foregoing, it is clear that, on the one hand, there are easily moldable materials (such as polystyrene and polyvinyl chloride) which are suitable for use in low cost, mass production of microtest plates and other sample-holding vessels, but which exhibit a substantial level of native fluorescence to create a problem in measuring the fluorescence of test samples. On the other hand, there are materials such as quartz and certain kinds of glass which have low levels of native fluorescence to avoid the foregoing problem, but which are unsuitable for the low cost production of microtest plates

and strips.

With the foregoing in mind, the general aim and purpose of this invention is to provide a novel, low cost, sample-holding vessel in which the native fluorescence of the material used to form the vessel is effectively suppressed or reduced to enhance the fluorometer's signal-to-noise ratio in a fluorometric measurement.

By suppressing the vessel's native fluorescence, virtually any desired material may be used for forming the vessel, including those which are used to make present day microtest plates and strips. With this invention, microtest plates and strips may therefore be manufactured with their customary materials (such as polystyrene or polyvinyl chloride) at low, affordable costs without encountering the problem associated with the material's native fluorescence. Vessels made in accordance with this invention are therefore suitable both for preparing the test sample and for holding the prepared test sample during the fluorometric measurement.

The foregoing object is achieved by providing the vessel with a barrier which blocks any significant penetration of the fluorometer's exciting light into the body of the sample-holding vessel, thereby preventing the vessel's material from being excited during the fluorometric measurement. When exposed to the fluorometer's exciting light, therefore, the vessel itself will not emit any significant light to interfere with the fluorometric measurement of the light emissions from the excited test sample. The signal-to-noise ratio in the fluorometer's detector is therefore improved to improve the accuracy of the fluorometric measurement.

In the illustrated embodiment, the barrier is of the physical type which is non-chemically incorporated with the vessel's material and which therefore does not alter the chemical properties of the vessel's material. The barrier may be formed by dispersing a particulate, barrier-defining material throughout the body of the vessel or by coating a preformed vessel with a barrier-defining material.

In the illustrated embodiments, the material used to form the barrier is a pigment which is opaque or substantially opaque to the wave length of the fluorometer's exciting light source and which is non-fluorescent or at least exhibits low fluorescence at the wave length of the light emitted from the excited test sample.

Black pigment is especially suitable for forming the barrier in the sample-holding vessel of this invention. It is opaque to and absorbs all light wave lengths. A plastic vessel containing a black pigment barrier exhibits virtually no detectable light emission when subjected to light in the ultraviolet range.

White pigment is also highly suitable for forming the barrier in the sample-holding vessel of this invention. While a vessel containing a white pigment barrier will fluoresce to some extent when exposed to ultraviolet light, the white pigment has the effect of appreciably strengthening the light emission produced by exciting the test sample, thereby improving the signal-to-noise ratio by both strengthening the signal of interest (the wave length of the light emitted by the excited test sample) and reducing the objectionable noise signal (the spurious light emission from the vessel itself).

Apart from white and black pigments, pigments of certain colors may also be suitable as long as they are opaque to the wave lengths of the fluorometer's exciting light and are non-fluorescent or exhibit low fluorescence at the wave length of the light emitted by the excited test sample.

Instead of using a pigment coating, a suitable metal such as silver may be deposited on appropriate surfaces of a preformed vessel to form the barrier that is opaque to the fluorometer's exciting light.

It will be appreciated that the vessel incorporating the principles of this invention is advantageously molded by low cost mass production techniques. Furthermore, the vessel incorporating the principles of this invention may be of any desired type such as a microtest plate, a microtest strip of wells, individual wells themselves, cuvettes and test tubes.

In order that the invention may be more readily understood, reference will now be made to the accompanying drawings, in which:-

Figure 1 is a perspective view of a 96 well microtest plate incorporating the principles of this invention;

Figure 2 is an enlarged, fragmentary section taken substantially along lines 2-2 of Figure 1;

Figure 3 is an enlarged fragmentary section similar to Figure 2, but illustrating another embodiment of this invention;

Figure 4 is a perspective view of a microtest strip of wells;

Figure 5 is an enlarged fragmentary section taken substantially along lines 5-5 of Figure 4;

Figure 6 is an enlarged fragmentary section similar to Figure 5, but illustrating the coated barrier embodiment;

Figure 7 is a schematic view of a fluorometer which is adapted for use with the sample-holding vessels of this invention;

Figure 8 is an enlarged fragmentary section similar to Figure 3, but illustrating a modification of the barrier-defining coating;

Figure 9 is an enlarged fragmentary section similar to Figure 3, but illustrating another modification of the barrier-defining coating;

Figure 10 is an enlarged fragmentary section similar to Figure 2, but illustrating a modification in which black pigment particles are dispersed in separately formed bottom walls for the microtest plate; and

Figure 11 is an enlarged fragmentary section similar to Figure 3, but illustrating another modification in which white pigment particles are dispersed in separately formed wells for the microtest plate.

For convenience, the term "vessel" is used in this specification (including the claims) to mean microtest plates, strips of wells, individual wells, cuvettes, test tubes or any other receptacle for holding a liquid. Also, the term "light" as used in this specification refers to both non-visible light (e.g., ultraviolet light) and light which is visible to the naked eye.

Referring to Figures 1 and 2, a molded, one-piece, rectangular microtest plate 20 incorporating the principles of this invention is integrally formed with a flat top wall 22 and side walls 24 depending from top wall 22 to form a depending skirt along all four sides of the plate to support the plate on a flat horizontal surface. Plate 20 is additionally formed with a multiplicity of precisely dimensioned, upwardly opening wells (or cups as they are also called) 26 for holding microliter quantities of liquid test samples or other solutions.

Wells 26 are integral with and open at top wall 22. By this construction, wells 26 are integrally joined to each other through top wall 22 and, unlike the embodiment shown in Figure 4, are not intended to be separated from each other.

As best shown in Figure 2, wells 26 depend from top wall 22 to lie in the region delimited by side walls 24. Wells 26 are uniformly spaced apart from each other and are uniformly dimensioned. Each of the wells 26 is formed with a cylindrical side wall 28 and a suitable bottom 30. The thicknesses of the wells' side and bottom walls are essentially uniform and are relatively small. The longitudinal axes of wells 26 are parallel and normally intersect top wall 22. In the embodiment illustrated in Figures 1 and 2,

spaced apart rows of wells with eight wells in each row to provide the standard total of 96 wells.

The foregoing type of microtest plate is essentially the same as the one described in U.S. Patent No. 3,356,462 which issued to N. M. Cooke et al on December 5, 1967. The disclosure in Patent No. 3,356,462 is incorporated into this specification by reference.

Plate 20 is preferably formed from commercially available polystyrene or polyvinyl chloride for use in immuno-assays. For example, the polystyrene may be any one of the following: Gulf SMD 3500, Foster Grant 9100D, Monsanto Lustrex 777 or Dow Chemical clear general purpose 666U. Plate 20 may alternatively be formed from any one of a number of other moldable materials such as acrylonitrile - butadiene - styrene and related multipolymers, acetal and homopolymer and copolymers thereof (Delrin for example), acrylics such as Plexiglass, allyl esters, amino resins, cellulosic plastics, epoxies, certain fluoroplastics, furan resin, ionomers, nitrile resins, phenolics, modified phenylene oxide, polyamide (nylon), polyamide - imide, polybutylene, polycarbonate (Lexan for example), polyester derivatives, polyethylene and ethylene copolymers, ethylene - vinyl acetate, polyimide, polymethylpentene, polyphenylene sulfide, polypropylene, derivatives of polystyrene, polyurethane, vinyl and polyvinyl copolymers other than polyvinyl chloride, silicone, styrene - acrylonitrile, sulfone polymers, vinylidene chloride and polymers and copolymers thereof, and alloys of the above.

The plastic material selected for plate 20 is required to be chemically compatible with the assay substances

to be used so as not to upset or interfere with the reactions which take place in assays, particularly immunoassays. In general, the particular application (e.g., immunoassays) determines the material to be used for making plate 20.

In accordance with this invention, pigment particles 32 are uniformly distributed or dispersed throughout plate 20 in sufficient quantity or density to form a barrier 34 which blocks any significant penetration of a fluorometer's exciting light or irradiation into plate 20, thereby preventing any significant excitation of the plate's plastic material during a fluorometric measurement. The pigment-defining barrier therefore has the effect of suppressing the native fluorescence of the plate's plastic material.

It will be appreciated that only the layer of pigment 32 lying immediately adjacent to the exposed surfaces of plate 20 defines the barrier for blocking penetration of the fluorometer's exciting light into plate 20. At best, therefore, only the plastic molecules lying on the plate's exposed surfaces will be fluoresced when exposed to a fluorometer's exciting light. The number of plastic molecules which will be fluoresced will consequently be reduced to a negligible number.

Pigment particles 32 are required to be opaque or at least substantially opaque at least to the wave length range of the fluorometer's exciting light. In addition, pigment particles 32 are require to be non-fluorescent or at least exhibit low fluorescence (which is less than that of the plastic material used to form plate 20) at the wave length of the light emissions resulting from excitation or irradiation of the test samples in wells 26.

Black and white pigments are especially suitable for defining the desired barrier previously mentioned. Other colored pigments such as green and red may also be used for defining barrier 34 for blocking penetration of the fluorometer's exciting light, so long as they are opaque at least to the wave length range of the fluorometer's exciting light and are non-fluorescent or at least exhibit low fluorescence at the wave lengths of the light emissions which result from exictation of the test samples.

A colored pigment other than white will reflect the particular wave length of its color so that the selection of a colored pigment other than white will afford selective enhancement of certain light wave lengths. For example, a red pigment will reflect the red wave length while abosorbing all other wave lengths in the light spectrum.

Black pigment, of course, absorbs all wave lengths in the visible and non-visible light spectrum and will therefore reflect none, while white pigment reflects all wave lengths in the spectrum, while absorbing none. It will be appreciated that the reflection of light produced by white pigment or other colored pigments differs from, and therefore should be distinguished from, light emissions originating from the native fluorescence of the material due to irradiation or excitation by an exciting light or radiation, which is usually in the ultraviolet range.

The pigment selected for establishing barrier 34 in plate 20 is required to be chemically compatible with the substances to be placed in wells 26 so that it does not alter the substances themselves or upset the reactions in assays which

are carried out using plate 20. It will be appreciated that pigment particles 32 are merely mixed with the plastic material of plate 20 and therefore are not chemically combined with the plate's plastic material. Pigment particles 32 therefore do not alter the chemical properties of the plate's plastic material.

In order to form barrier 34, it is not necessary that pigment particles 32 be uniformly dispersed throughout plate 20. Instead, they need only be dispersed to an adequate extent to form the desired barrier for blocking penetration of a fluorometer's exciting light into the plate.

From the foregoing description it is evident that the amount of pigment particles 32 incorporated into plate 20 must be sufficient to prevent any significant penetration of a fluorometer's exciting light into the microtest plate to thereby suppress or at least reduce the native fluorescence of the plastic material used to form plate 20. On the other hand, the amount of pigment particles 32 used to form barrier 34 should not be so great as to compromise or impair the structural strength of plate 20 or to upset or adversely affect the molding conditions for molding plate 20 from a selected plastic.

In carrying out an embodiment of this invention, pigment particles 32 are added to and thoroughly mixed with the plastic material prior to molding of the plate, so that barrier 34 is effectively formed in situ in the course of molding plate 20 into its final form. A satisfactory barrier may be achieved by adding pigment to the selected plastic material in an amount ranging from about 0.01% to about 10% based on the total weight of the plate's materials (i.e., the weight of the plastic

material plus the weight of the pigment particles). Percentages of approximately 0.1% to approximately 1.0%, however, are preferred. The minimum amount of pigment particles will vary depending upon the type of pigment selected and also on the particular applications which plate 20 will be used for. The limit on the maximum amount of pigment will, as previously noted, depend primarily on the desired structural strength of plate 20 or, for that matter, any other vessel which is formed with the pigment-defining barrier of this invention.

In one preparation for the microtest plate for use in fluorescent immunoassays, commercially available carbon based black pigment (PMS 4500 SUDC, which is pure carbon and which is supplied by Plastic Molding Supply, a company located in Brunswick, New Jersey) was mixed with commercially available clear polystyrene in an amount equal or proportional to 0.22 pounds (100 grams) of the black carbon pigment per 100 pounds of clear polystyrene. The amount of pigment present in the mixture therefore amounted to slightly less than 0.22% of the total weight of the mixture or exactly 0.22% of the weight of the total weight of the polystyrene. Following mixing to uniformly disperse or distribute the pigment throughout the plastic, microtest plates of the type shown in Figure 1 were injection molded from the pigment-plastic mixture. Microtest plates produced from this mixture were observed to have a black, opaque appearance.

When exposed to an exciting light in the ultraviolet range, plates made from the foregoing black pigment-polystyrene formulation exhibited no detectable light emissions or, at best, only negligible light emissions when exposed to an exciting light in the ultraviolet range. Accordingly, the black-

pigmented microtest plates described in the foregoing example have the effect of dramatically increasing the signal-to-noise ratio of a fluorometer's light intensity detector.

In another example, commercially available white pigment (Plastic Molding Supply's PMS 350 SUDC) was mixed with the same polystyrene used in the preceding example in the amounts specified in the first example for black pigment (i.e., 0.22 lbs. of white pigment per 100 lbs. of polystyrene). Microtest plates injection molded from the white-pigmented mixture were found to reduce the intensity of the vesssl's fluorescently produced light emissions to almost one-half the value measured for conventional clear polystyrene microtest plates without the pigment-defining barrier of this invention.

Although not reducing the light emissions due to native fluorescence as much as black pigment, the white pigment was found to have the advantageous effect of substantially increasing the intensity of the light emissions resulting from the fluorescent excitation of test samples in the plate's wells, thereby strengthening the signal of interest due to the light emitted by the excited test samples. Accordingly, white pigment has the effect of improving the signal-to-noise ratio in two ways, one by strengthening the signal of interest, and the other by reducing the noise signal due to the plate's native fluorescence. The increase in the intensity of the light emitted by the fluorescently excited test sample in the white-pigmented microtest plate is believed to be due to the high reflection property of white pigment which causes the fluorometer's exciting light and also the emitted light (due to the fluorescent excitation of the test sample) to be reflected from the surfaces of the sample-

receiving wells 26 in the microtest plate. This reflection appears to cause the exciting light and the emitted light to move or bounce back and forth through the sample to increase the intensity of the light emitted by the fluorescently excited sample.

Another white pigment considered to be suitable for forming barrier 34 is Dupont's Ti-Pure R-101 CFKD, which is titanium dioxide. It is evident that any other type of white or black pigment may be used so long as it is opaque or at least substantially opaque to the wave length of the fluorometer's exciting light and is non-fluorescent or at least exhibits low fluorescence at the wave length of the light emitted by the fluorescently excited test sample.

Instead of dispersing the barrier-defining pigment particles 32 into the plate's plastic body, barrier 34 may be established by applying a thin coating or layer of a pigment-containing coloring agent 36 to the exposed surfaces of a pre-formed microtest plate 20a and especially to those surface areas which will be exposed to the fluorometer's exciting light as shown in Figure 3.

Plate 20a has the same configuration as plate 20 and is of the same construction as plate 20 except that coating 36 is applied in lieu of dispersing the pigment particles throughout the plastic body of the plate in the manner shown in Figure 2. The body of plate 20a is therefore devoid of pigment particles and is formed entirely from a suitable plastic material such as polystyrene or polyvinyl chloride. To the extent that plates 20 and 20a are the same, like reference numerals have been applied to designate like parts, except that

the reference numerals used for plate 20a have been suffixed by the letter "a" to distinguish them from those used for plate 20.

The coloring agent used for coating 36 has three major ingredients, namely the pigment, a vehicle for the pigment, and a solvent. When the coloring agent is applied to form coating 36 it is clear that the solvent flashes off as the coating drys, leaving only the pigment and its vehicle. Any suitable vehicle may be used such as polystyrene resin. Any suitable solvent may be used such as methylene chloride. Finally, any suitable pigment may be used so long as it is opaque or at least substantially opaque at least to the wave length of the fluorometer's exciting light and is non-fluorescent or at least exhibits low fluorescence (significantly less than that of the plastic material used to mold plate 20a) at least at the wave length of the fluorescently excited test sample. For example, a black pigment or a white pigment may be used as described above in the preceding examples. Alternatively, a colored pigment may be used, such as red, so long as it is opaque to the wave length of the fluorometer's exciting light and is non-fluorescent or at least exhibits low fluorescence at least at the wave length of the light emission resulting from the fluorescent excitation of the test samples.

The coloring agent selected for forming coating 36 is required to be chemically compatible with the substances placed in wells 26a so that it does not alter the substances themselves or upset the reactions in assays which are carried out in plate 20a. The coloring agent may be applied to plate 20a by spraying or any other suitable means.

In the embodiment shown in Figure 3, it will be appreciated that plate 20a is first molded or otherwise formed from a suitable plastic. Thereafter, the coloring agent is applied to the appropriate surfaces of the plate to form the barrier-defining coating 36 which prevents penetration of the fluorometer's exciting light into the plate's plastic body.

In the embodiment of Figure 3, coating 36 is shown to cover the entire top surface of top wall 22 and all of the interior surfaces of wells 26. The barrier formed by coating 36 therefore prevents penetration of a fluorometer's exciting light beam into the plastic body of plate 20a to thereby prevent plate 20a from being fluorescently excited by the exciting light. The exciting light in this embodiment and the embodiment of Figures 1 and 2 is directed through the open end of each of the plate's wells. To conduct a fluorometric measurement it therefore will be appreciated that a fluorometer of the frontal approach type is required such as the one schematically shown in Figure 7.

In Figure 7, the fluorometer comprises a suitable light source if source of radiation 40 for supplying an exciting light or radiation which may vary from about 200 nanometers to about 700 nanometers depending upon the material to be fluoresced and which is usually in the ultraviolet wave length range

The exciting light beam developed by source 40 is indicated by arrow 42 and is directed through a filter 44 and a slit or aperture 46 to a partially silvered mirror 48. Mirror 48 is located at an acute angle to the direction of the exciting light from source 40 to reflect the exciting light beam

downwardly through the open upper end of a sample holding vessel so that it impinges directly on and penetrates the test sample in the vessel before impinging any of the vessel's surfaces. A shutter 50 may optionally be located between filter 44 and slit 48. The beam of exciting light 42 may be focused or suitably targeted so that upon being reflected by mirror 48 it will enter the vessel through the vessel's open upper end. Because mirror 48 is only partially silvered, part of the exciting light will pass through the mirror and will therefore be wasted as indicated by the arrow 52.

Upon being exposed to the exciting light, the test sample (indicated at 54 in Figure 7) will be fluorescently excited to emit light at a predetermined wave length or range of wave lengths. The wave length of the emitted light depends on the particular substance which is fluorescently excited and is typically in the range extending from about 350mm to about 900mm. The emitted light produced by the excitation of test sample 54 will pass directly upwardly through the open upper end of the vessel and will pass through the partially silvered mirror 48 as indicated by arrow 56. Part of the emitted light will be reflected by the partially silvered mirror and will therefore be wasted.

The emitted light 56 passing through mirror 48 will pass through a further filter 58 (which may be of the bandpass type to pass just the wave length of interest) and another slit or aperture 60 to a detector 62 which measures the intensity of the emitted light. A device such as a meter 64 is connected to detector 62 and supplies a read-out of the intensity of the

emitted light detected in detector 62 to provide a fluorometric measurement of the intensity of the emitted light and, therefore, the fluorescence of the fluorescently excited substance in test sample 54.

Another frontal approach type of fluorometer which is suitable for measuring the fluorescence of samples in wells 26, 26a is described in our co-pending EPC Application (corresponding to U.S. Patent Application No. 433825) filed on even date herewith.

From the foregoing description it will be appreciated that the beam of exciting light 42 enters through the open upper end of the sample-holding vessel, and that the light emission due to the fluorescent excitation of sample 54 exits through the open upper end of the vessel. The provision of barrier 34 in the form shown in Figure 1 or in the form shown in Figure 3 therefore does not interfere with the entrance of exciting light or the exit of emitted light.

It is evident from the foregoing that any suitable frontal approach type of fluorometer may be used with sample-holding vessels containing the barrier of this invention. The particular type of frontal approach fluorometer therefore does not constitute a part of this invention.

In Figures 4 and 5, the pigment-defining barrier of this invention is shown to be incorporated into a molded, one-

piece microtest strip 70 containing a straight row of parallel, upwardly opening wells 72 for containing microliter quantities of liquid. In this embodiment, each of the wells 72 is formed with a cylindrical side wall 76 and a flat bottom wall 78 as shown in Figure 5.

Each of the wells 70 is integrally joined to the adjacently disposed wells by frangible stem-like segments 74 which may take the form of mold flashings. Segments 74 lie flush with the lips or open upper ends of wells 72. Segments 74 are easily broken by hand to enable one or more of the wells 72 to be separated from the strip. One of the disconnected wells 72 is shown in Figure 7.

Wells 72 are provided with precise, uniform dimensions. The longitudinal axes of wells are parallel and contained in a common plane.

The previously mentioned Thorne patent discloses a matrix of wells which are integrally joined together by frangible stems for separation into individual wells and also into strips of the type shown in Figure 4. It will be appreciated that the principles of this invention may be applied to the well structure described in the Thorne patent. The disclosure of the Thorne patent is hereby incorporated into this specification by reference.

Strip 70 is preferably formed from polystyrene or polyvinyl chloride. Alternatively, strip 70 may be formed from any of the other moldable materials mentioned for plates 20 and 20a.

Where strip 70 is formed from polyvinyl chloride, the individual wells 72 may be integrally joined together by a thin severable segment portion (not shown) which can easily be cut with scissors.

A holder of the type shown and described in the Thorne patent may be used to support strip 70 and/or the individual wells. Alternatively, any other suitable holder may be used to support the strip while conducting an assay. If desired the holder itself may be provided with the barrier of this invention.

Like the embodiment shown in Figure 2, pigment particles 32 are uniformly distributed or dispersed throughout strip 70 in sufficient quantity or density to form the required barrier 34 which blocks any significant penetration of the fluorometer's exciting light or radiation into strip 70, thereby preventing any significant excitation of the strip's plastic material during a fluorometric measurement. The pigment-defining barrier for strip 70 therefore has the effect of supressing the native fluorescence of the strip's plastic material in the same manner described for the embodiment of Figure 2.

The pigments used to form the barrier in the embodiment of Figure 2 are intended for use to form the barrier in strip 70. Like Figure 2, black or white pigment is especially suitable for forming the barrier in strip 70. The amount of pigment used to form the barrier in strip 70 is the same as that described for the embodiment in Figure 2.

In manufacturing strip 70, it is evident that pigment particles 32 are first thoroughly mixed with the plastic material prior to molding strip 70. Thereafter, strip 70

is molded from the mixture of the plastic material and pigment particles so that the pigment particles are captured or suspended in the solidiifed, molded plastic body to define the barrier 34.

Instead of dispersing the barrier-defining pigment particles 32 into the strip's plastic body, the barrier 34 may be established by applying a thin coating or layer of pigment 36a to the exposed surfaces of a preformed microtest strip 70a and especially to those surface areas which will be exposed to the fluorometer's exciting light as shown in Figure 6. Strip 70a has the same configuration as strip 70 and is of the same construction as strip 70 except that the coating 36a is applied in lieu of dispersing the pigment particles throughout the strip's plastic body. The body of strip 70a is therefore devoid of pigment and is formed entirely of a suitable moldable material such as polystyrene or polyvinyl chloride. To the extent that strips 70 and 70a are the same, like reference numerals have been applied to designate like parts, except that the reference numerals used for strip 70a have been suffixed by the letter "a" to distinguish them from those used for strip 70.

Coating 36a is the same as coating 36 and may be formed from those pigments used for coating 36, such as white or black. Coating 36a is applied to the interior surfaces of well 72a. It may optionally be applied to the upwardly facing surface of strip 70a as shown. Covering the upwardly facing surface of strip 70a with coating 36a, however, is not necessary.

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Crystallite Size Distributions from Intensities of Diffraction Spots

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Abstract

The diffraction patterns of coarse grained materials are compared to specimens with crystallite sizes of less than 1 μm characterized by diffraction spots. Based on an analysis of spot intensities it is shown that for: (a) an at-random distribution of crystallite orientations and (b) under the assumption of specific crystallite shapes it becomes possible to evaluate the distribution $N(\ell)$ of linear crystallite dimensions ($0.1\ \mu m < \ell < 20\ \mu m$) from measured spot intensity distributions z(i).

Concept of Stephen and Barnes

This concept may be discussed as an introduction. Four basic assumptions are used:

1. All crystallites of the ensemble under investigation have identical size.
2. There occurs no remarkable self absorption within these crystallites and therefore the diffracted intensity is directly proportional to the crystallite volume.
3. All crystallites are oriented completely at random and
4. All crystallites are of cubic shape.

With the crystallite volume v, the number N of crystallites per cm^3 becomes $N = 1 / v$. The incident beam of monochromatic X-rays impinges perpendicularly on the flat specimen surface; its cross section is q and its divergence $\Delta\theta$. Further X-ray characteristics are Bragg angle θ and multiplicity factor p. Considering a volume $q \cdot dt$ of thickness dt a number dz of crystallites favorably oriented for reflection are to be expected

$$dz = N \cdot q \cdot dt \cdot \frac{p \cdot \cos\theta \cdot \Delta\theta}{2} \qquad (1)$$

Because the unattenuated diffracted intensity i_0 from a single crystallite is described by $I_0 \cdot \chi \cdot v$ (I_0 being the beam intensity on the specimen surface, and χ, the proportionality factor), the intensity i of a beamlet diffracted by a crystallite in depth t is

$$i = i_0 \cdot e^{-\tilde{\mu} \cdot t \cdot (1 - 1/\cos 2\theta)} \qquad (2)$$

($\tilde{\mu}$ being the linear absorption coefficient). Within the volume $q \cdot dt$ the depth varies from to to $t + dt$, and according to Equation 2 the spot intensity varies from i to $i + di$ ($di < 0$). When expressing dt in Equation 1 by di from Equation 2, dz/di can be written as follows:

$$\frac{dz}{di} = -N \cdot q \cdot \frac{1}{\tilde{\mu} \cdot (1 - 1/\cos 2\theta)} \cdot \frac{p \cdot \cos\theta \cdot \Delta\theta}{2} \cdot \frac{1}{i} \qquad (3)$$

and $\Delta z = (dz/di)$. Δi describes the number of diffraction spots on the Debye-Scherrer ring, with spot intensities ranging from i to $i + \Delta i$. Integration of Δz from i to i_0 delivers the number $z = z(i)$ of all diffraction spots with intensities greater than i ($i < i_0$):

$$z = N \cdot q \cdot \frac{1}{\tilde{\mu} \cdot (1 - 1/\cos 2\theta)} \cdot \frac{p \cdot \cos\theta \cdot \Delta\theta}{2} \cdot \ln \frac{i_0}{i} \qquad (4)$$

As mentioned above (Equation 2) i_0 is the maximum possible intensity of diffraction spots. Equation 3 or 4 can be used for the determination of N: $v = 1/N$ and the edge length $\ell = v^{1/3}$ of the cubic particles. The length ℓ represents the crystallite size.

Crystallite Size Distribution

Since spot intensities are measured, and are directly proportional to the crystallite volume, crystallite volume distributions N(v) should be used instead of crystallite size distributions $N(\ell)$. A correlation between these distribution functions is obtained from

$$N(v) \cdot dv = N(\ell) \cdot d\ell \qquad (6)$$

for given crystallite shapes. A cubic shape ($v = \ell^3$) delivers

$$N(\ell) = 3 \cdot v^{2/3} \cdot N(v) \qquad (7)$$

and a spherical shape ($v = \chi \cdot \ell^3/6$) delivers

$$N(\ell) = 1.92 \cdot v^{2/3} \cdot N(v) \qquad (8)$$

Because in 1 cm^3 of the specimen the number of crystallites with volumes from v to $v + dv$ is $N(v) \cdot dv$, the total number N of crystallites per cm^3 is

$$N = \int_{v_{min}}^{v_{max}} N(v) \cdot dv \qquad (9)$$

and the volume must become 1 cm^3:

$$1 = \int_{v_{min}}^{v_{max}} N(v) \cdot v \cdot dv, \qquad (10)$$

v_{min} and v_{max} being the smallest and largest crystallites. As a crystallite distribution exists, there is no longer $v = 1/N$ (Equations 9 and 10). 1/N defines now an averaged crystallite volume v_{av}.

The concept of Stephen and Barnes (1937) can be seen as a special case of a general concept based on crystallite volume distributions. N(v) has to be replaced by a delta function and for the following treatment of the problem Equation 3 is rewritten

$$N = -\frac{2 \cdot \tilde{\mu} \cdot (1 - 1/\cos 2\theta)}{q \cdot p \cdot \cos\theta \cdot \Delta\theta} \cdot \frac{dz}{di} \cdot i \qquad (11)$$

An evaluation of a diffraction pattern delivers a response of z versus i. Spot intensities are measured in arbitrary units. From Equation 11 it can be learned that for $N(v) = \delta_v$, the product of the derivative dz/di and is is constant and moreover does not depend on the units chosen for the i-scale. After these introductory remarks the influence of a crystallite volume distribution on measured distributions of diffraction spots should be outlined.

A diffracting crystallite of volume v(0) on the surface is observed by an intensity i

$$i = I_0 \cdot \chi \cdot v(0). \qquad (12)$$

An identical intensity from a crystal in depth t is only obtained, if the volume of this crystal is

$$v(t) = v(0)\cdot e^{\bar{\mu}\cdot t\cdot(1 - 1/\cos 2\theta)}. \quad (13)$$

A larger crystal of volume $v(t) > v(0)$ in depth t thus contributes to the number of observed diffraction spots with intensity i, and consequently the largest crystal, v_{max} of N(v), is detected by i as it is located in depth t_{max}:

$$t_{max} = \frac{1}{\bar{\mu}\cdot(1 - 1/\cos 2\theta)} \cdot \ln \frac{v_{max}}{v(0)}. \quad (14)$$

Since $N(v)\cdot dv$ is the number of crystallites with volumes within v and v + dv, an intensity from i to i + di has to be considered and the total number dz of spots with an intensity within i and i + di follows from the sum of

$$d^2z(0) = N[v(0)] \cdot dv\cdot q\cdot dt\cdot \frac{p\cdot\cos\theta\cdot\Delta\theta}{2}$$

plus

$$d^2z(t_1) = N[v(t_1)] \cdot dv\cdot q\cdot dt\cdot \frac{p\cdot\cos\theta\cdot\Delta\theta}{2} \quad (15)$$

$$d^2z(t_{max}) = N[v(t_{max})] \cdot dv\cdot q\cdot dt\cdot \frac{p\cdot\cos\theta\cdot\Delta\theta}{2}$$

which is

$$dz = q\cdot dt\cdot\frac{p\cdot\cos\theta\cdot\Delta\theta}{2}\cdot\int_{v_{min}}^{v_{max}} N(v)\cdot dv. \quad (16)$$

From

$$i = I_o\cdot\chi\cdot v\cdot e^{-\bar{\mu}\cdot t\cdot(1 - 1/\cos 2\theta)} \quad (17)$$

follows

$$di = -i\cdot\bar{\mu}\cdot(1 - 1/\cos 2\theta)\cdot dt, \quad (18)$$

and when employing dt from Equation 18 for Equation 16 one obtains a result comparable to Equation 11:

$$\int_{v_{min}}^{v_{max}} N(v)\cdot dv = -\frac{2\cdot\bar{\mu}\cdot(1 - 1/\cos 2\theta)}{q\cdot p\cdot\cos\theta\cdot\Delta\theta}\cdot\frac{dz}{di}\cdot i \quad (19)$$

and v(0) and i are correlated by Equation 12. It means that the intensity of the diffraction spots defines the range of integration. A decrease of i decreases linearly the volume v, and as i is equal to i_{min}

$$i_{min} = I_o\cdot\chi\cdot v_{min} \quad (20)$$

or even lower; the integral of Equation 19 becomes identical with Equation 9. Therefore, for the low i-range the response described by the right side of Equation 19 versus i, behaves identically to the response according to Equation 11, or in other words, all information on distribution functions comes exclusively from the high i-range of diffraction spots.

Figure 1 depicts the response of the right side of Equation 19 (it is y) in dependence on i for a delta function (Stephen and Barnes, 1937) and a crystallite volume distribution. Since N of the delta function is in general not identical to N of Equation 9 different values have been chosen. A delta function is identified by its rectangular shape, whereas the distribution function is split up into two portions, a constant (low i –) portion identical to the delta function and its typical integral (high i –) portion. The negative derivative of y(i) is a distribution n(i).

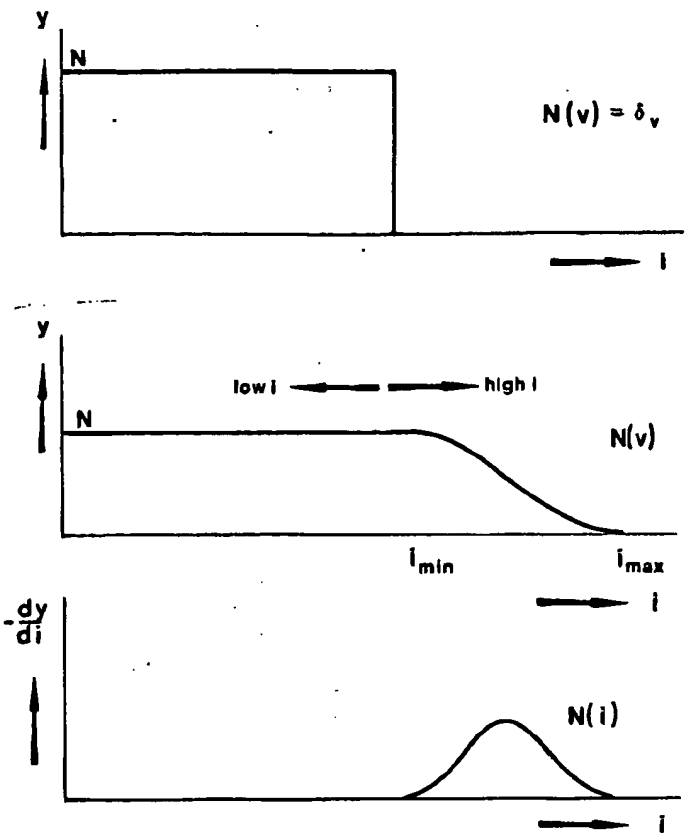

Fig. 1

Figure 1: y(i)-responses for only one crystal size and a distribution N(v) and the derivative -dy/di of the second y(i)-response.

Determination of N(y) and N(ℓ)

As mentioned, the intensity is given in arbitrary units. When replacing i by k.i the product of $(dz/di)\cdot i$ becomes $(dz/d(k\cdot i))\cdot k\cdot i$, which is identical to $(dz/di)\cdot i$. Consequently, the choice of the i-scale does not influence the result of Equation 19.

A first conclusion of this statement is that the number N of crystallites per cm^3 is identical to the value of the constant response in the low i-region of Figure 1.

The variable i is given by i = const.v (see Equation 12). The negative derivative of y(i) is N(i) = – dy/di. With this definition, N(v) and N(i) are correlated by a linear equation.

$$N(i) = N(v)\,(1/\text{const}) \quad (21)$$

Integration of N(i) gives

$$\int_{i_{min}}^{i_{max}} N(i)\cdot di = \int_{v_{min}}^{v_{max}} \frac{N(v)}{\text{const}}\cdot\text{const}\cdot dv = N. \quad (22)$$

It confirms the above given first conclusion, but does not enable determination of the unknown value of const. Using only the second normalization (Equation 10) one obtains

$$\int_{i_{min}}^{i_{max}} N(i)\cdot di = \int_{v_{min}}^{v_{max}} \frac{N(v)}{const}\cdot(const\cdot v)\cdot(const\cdot dv) = const \qquad (23)$$

As the ordinate scale is transformed from y to y·const and the abscissa scale from i to i/const, N(i) is transformed into N(v) and finally Equation 7 or Equation 8 has to be used in order to describe the original goal of these considerations, namely the crystallite size distribution N(ℓ).

Experimental Verification

An estimation should help to quantify the practicable crystallite size range. Small crystallites of 10 to 100 nm are indicated by line broadening. The formula from Scherrer (1918),

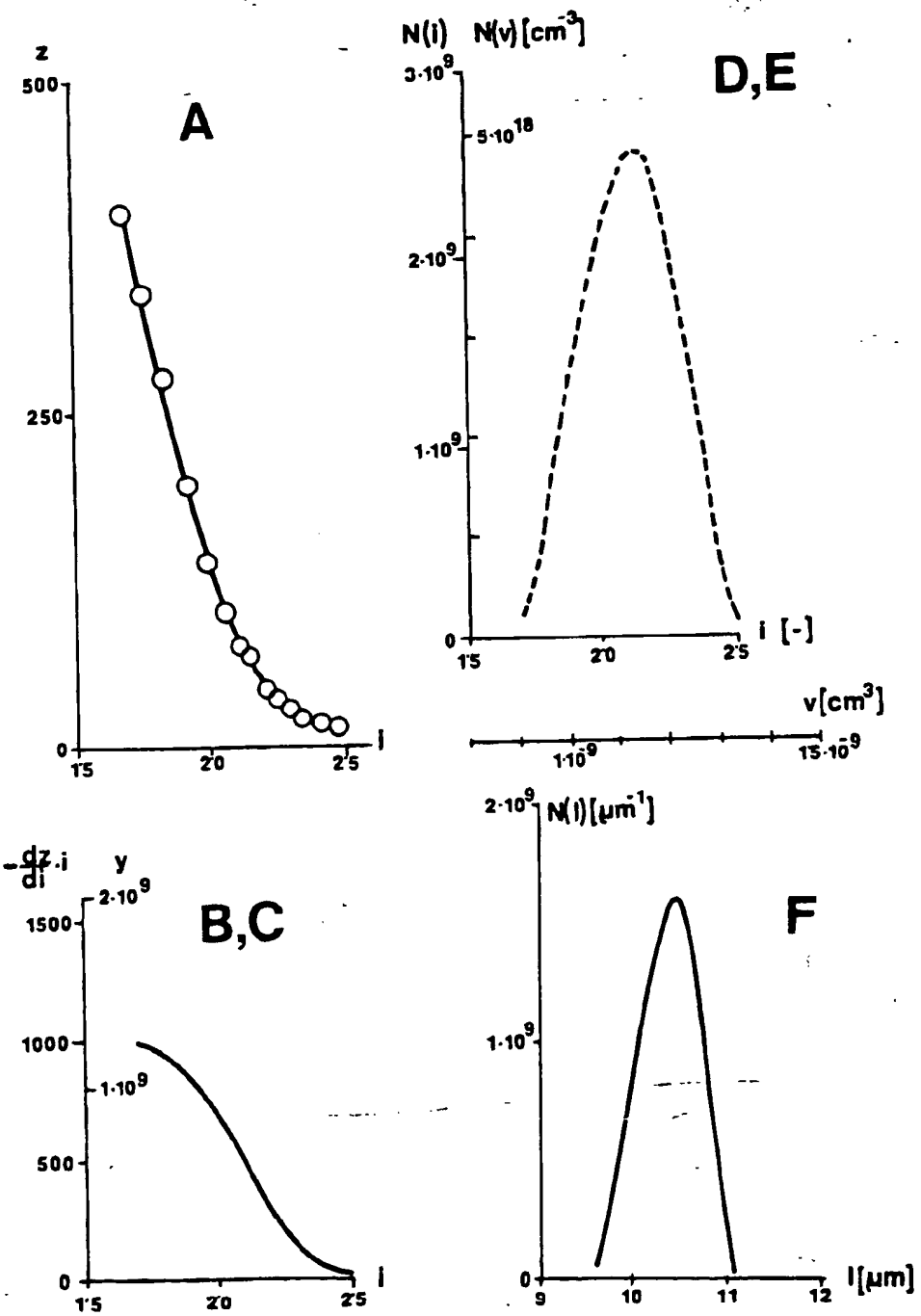

Figure 2: A) Experimental z(i)-response
B) (-dz/di)·i as obtained from (A) in dependence on i
C) y(i)-response (y = 1.21 · 10⁶ · (-dz/di)·i)
D) N(i) = (-dy/di) as obtained from (C)
E) With const·N(i) and i/const follows N(v)
F) N(v) has been transformed to the crystallite size distribution N(ℓ) under the assumption of a cubic crystallite shape.

$$B = 0.9\,\lambda/l\cdot\cos\theta,$$

delivers as an example, for a crystallite size ℓ = 100 nm, with wavelength λ = 1.5 Å of X-radiation at Bragg angle θ = 70°, the broadening, B, equals $3.95\cdot10^{-3}$ radians of the diffraction line, due to crystallite size. With the detector at a distance of 5 cm from the crystallite, the broadening is about 0.2 mm. Further details of this Debye-Scherrer ring are diameter d = 2.5.cos (180-2θ) = 7.66 cm and its width which may be 1 mm, due to spectral width of X-radiation, beam divergence, entrance slit width and crystallite size. Consequently, this ring covers an area of 241 mm^2. A single crystallite contributes to the ring a spot of a dimension determined only by crystallite size and by spectral width, which is estimated to be $\Delta\lambda/\lambda = 4.10^{-4}$. From the spectral width the contribution to spot dimensions is 0.05 mm. A rough estimation thus delivers spot diameters of $\sqrt{0.2^2+0.05^2}$ = 0.206 mm and a great number of spots distributed at random is responsible for the apparently continuously blackened Debye-Scherrer ring. What is the number of spots in our specific example? The observable intensity may cover a range from i down to i/100. It defines the depth t of information:

$$t = \frac{1}{\tilde{\mu}\cdot(1-1/\cos2\theta)}\cdot\ln\left(\frac{i}{i/100}\right) = 80\ \mu m,$$

when using $\tilde{\mu} = (\frac{\mu}{\rho})\cdot\zeta = 50.5 = 250\ cm^{-1}$. A diameter of 0.5 mm of the X-ray beam delivers a cross section q = 0.2 mm^2 and as the width of the ring is 1 mm, considering all broadening factors, $\Delta\theta$ must be $\sqrt{1^2-0.2^2-0.05^2-0.5^2}$ / 100 = $8.4\cdot10^{-3}$ radians. Assuming, finally, p = 8, a number, z, of crystallites favorably oriented for reflection – z is the unknown number of spots – is calculated from

$$z = \frac{1}{1^3}\cdot q\cdot t\cdot\frac{p\cdot\cos\theta\cdot\Delta\theta}{2} = \frac{1}{(1x10^{-4})^3}$$

$$\cdot0.2\cdot8x10^{-2}\,\frac{8\cdot\cos70\cdot8.4x10^{-3}}{2} = 1.84x10^8$$

A single spot covers an area of $0.206^2\pi/4 = 3.3x10^{-2}$ mm^2 and with the spots side by side only a maximum number of separated spots is possible, i.e., $241/3.3x10^{-2}$ = 7300. The dominating quantity for a reduction of the number of spots to this value is the crystallite dimension ℓ. An increase from 100 nm to 1 μm reduces z from $1.84x10^8$ to $1.84x10^5$ spots. A further order of magnitude, ℓ = 10 μm, is depicted by 184 spots on the ring, which are well separated. As a size range from 0.1 μm to 20 μm should be covered by this concept, further variables are found in the experimental arrangement. They are q^3 (Hirsch & Kellar, 1951; Kellar *et al*, 1950), $\Delta\theta$, and moreover, a proper choice of the X-radiation enabling variations of $\tilde{\mu}$, θ and p.

A similar concept has been published in earlier papers (Ebel, 1965; Ebel *et al*, 1967; Wagendristel *et al*, 1968). Gaussian and Lorentzian distributions have been assumed for crystallite size distributions and the experiments were dedicated to determination of halfwidth and position of these distributions. The reason for these reduced efforts to obtain just halfwidth and position, instead of detailed information on the real shape of size distributions, lies in the experimental possibilities (photographic registration, calibration of the blackening response and microphotometric evaluation). In the meantime 2D-position sensitive detectors are available; evaluations like background subtraction, integration of spot intensities and finally the calculations according to the concept given in this paper can be performed by microcomputers.

The need for crystallites oriented at random makes especially fine-grained powders an essential type of application. Because the powder grains are crystallites, the crystallite dimension is automatically the grain size. Thus, the method can be used for the determination of powder grain size distributions. These measurements and evaluations can now be performed; the software for a specific problem and an experimental setup have been developed, and are completely automated and fully competitive with sedimentation measurements.

Crystallites can contain crystal defects like dislocations, vacancies, interstitials or stacking faults and should have edgelengths, l, of $\mu\cdot\ell < 0.1$ in order to make sure that $1 \propto v$ holds.

The results of experiments performed on aluminum are depicted in Figures 2A through 2F. Experimental conditions: CuKα-radiation, Al[333] + [1.15], θ = 81.7°, μ/ρ = 51 cm^{2}g^{-1}, ρ = 2.7 gcm^{-3}, q = $d^2\pi/4$, d = 0.8 mm, p = 8 + 24, $\Delta\theta = 2.10^{-2}$.

From these data follows

$$\frac{2\cdot\mu\cdot\rho\cdot(1-1/\cos2\theta)}{q\cdot p\cdot\cos\theta\cdot\Delta\theta} = 1.21x10^6\ cm^{-3}$$

According to Equation 23 the response of N(i) from Figure 2D delivers

$$const = \int_{v_{min}}^{v_{max}} N(i)\cdot i\cdot di = 1.88x10^9$$

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